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SEROLOGIC AND MOLECULAR EVIDENCE FOR TESTUDINID HERPESVIRUS 2 INFECTION IN WILD AGASSIZ'S DESERT TORTOISES, *GOPHERUS AGASSIZII*

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ABSTRACT: Following field observations of wild Agassiz's desert tortoises (*Gopherus agassizii*) with oral lesions similar to those seen in captive tortoises with herpesvirus infection, we measured the prevalence of antibodies to Testudinid herpesvirus (TeHV) 3 in wild populations of desert tortoises in California. The survey revealed 30.9% antibody prevalence. In 2009 and 2010, two wild adult male desert tortoises, with gross lesions consistent with trauma and puncture wounds, respectively, were necropsied. Tortoise 1 was from the central Mojave Desert and tortoise 2 was from the northeastern Mojave Desert. We extracted DNA from the tongue of tortoise 1 and from the tongue and nasal mucosa of tortoise 2. Sequencing of polymerase chain reaction products of the herpesviral DNA-dependent DNA polymerase gene and the UL39 gene respectively showed 100% nucleotide identity with TeHV2, which was previously detected in an ill captive desert tortoise in California. Although several cases of herpesvirus infection have been described in captive desert tortoises, our findings represent the first conclusive molecular evidence of TeHV2 infection in wild desert tortoises. The serologic findings support cross-reactivity between TeHV2 and TeHV3. Further studies to determine the ecology, prevalence, and clinical significance of this virus in tortoise populations are needed.

Key words: Agassiz's desert tortoise, *Gopherus agassizii*, Testudinid Herpesvirus 2, Testudinid Herpesvirus 3, tongue.

INTRODUCTION

Herpesviruses are well-recognized pathogens of chelonians (Jacobson, 2000, 2007). Within Chelonia (extant turtles and tortoises), numerous reports of herpesvirus infection exist for members of the family Testudinidae (tortoises) and Cheloniidae (four of the five genera of sea turtles). Herpesvirus-like particles and lesions have also been reported from hosts in the family Emydidae (pond turtles), although no sequence characterization has been published.

Where sequencing data are available, all chelonian and other reptilian herpesviruses belong to the subfamily Alphaherpesvirinae (VanDevanter et al., 1996; Quackenbush et al., 1998; Teifke et al., 2000; Une et al., 2000; Stacy et al., 2008). The genus *Chelonivirus* has been proposed to include the monophyletic group of herpesviruses that have chelonian hosts (Stacy et al., 2008; Bicknese et al., 2010). Using molecular sequencing technology, four distinct genotypes of tortoise herpesviruses (THVs) are recognized: THV1 (Une et al., 2000; Murakami et al., 2001; Stöhr and Marschang, 2010), THV2 (Johnson et al., 2005), THV3 (Marschang et al., 2006; Bicknese et al., 2010), and THV4 (Bicknese et al., 2010). This enumeration is recent and can be found in Bicknese et al. (2010). We expect new genotypes of herpesviruses to be described as species of tortoises from different geographic regions are examined. We recommend use of the abbreviation TeHV for Testudinid herpesvirus. Hereafter, the four Testudinid

herpesviruses will be abbreviated: TeHV1, TeHV2, TeHV3, and TeHV4.

The first report of a herpesvirus-like agent associated with a lesion in an Agassiz's desert tortoise (*Gopherus agassizii*; hereafter, desert tortoise refers to *G. agassizii*, one of two identified species within the desert tortoise species complex [Murphy et al., 2011]) involved a 6-yr-old cachectic captive (Harper et al., 1982). Other papers describing herpesvirus-like particles in desert tortoises followed (Pettan-Brewer et al., 1996; Martinez-Silvestre et al., 1999). In a recent report (Johnson et al., 2005), a captive desert tortoise from California with a severe pharyngitis was found by light microscopy to have herpesvirus-like intranuclear inclusion bodies in mucosal epithelial cells. Using transmission electron microscopy, herpesvirus particles were detected in the mucosal epithelium; PCR amplification and sequencing identified herpesvirus nucleic acid. Sequence analysis determined this herpesvirus was TeHV2 and was distinct from TeHV1. This tortoise was housed with three other desert tortoises and one Texas tortoise (*Gopherus berlandieri*) for 5 mo prior to developing clinical signs of disease. Two of the desert tortoises and the Texas tortoise were positive for antiherpesvirus antibodies, while the remaining desert tortoise was negative. It is unknown whether these tortoises were infected with herpesvirus prior to captivity, if they were exposed to other infected tortoises at some point during captivity, or if they had the same point source as the infected tortoise. In 2000 and 2001, Johnson et al. (2006) conducted a serologic survey of 109 captive tortoises, most of which were desert tortoises from cities and towns in the Mojave Desert, California, and found that 26.6% of the tortoises were antibody-positive to a herpesvirus described from *Testudo* spp., now referred to as TeHV3.

The first indications of a possible herpesvirus in wild populations of desert tortoises appeared in the early 1990s, shortly after the desert tortoise was federally listed as a threatened species under the Endangered Species Act, as amended, in California, Nevada, Utah, and northwestern Arizona (US Fish and Wildlife Service, 1990). The appearance of newly emerging diseases was one reason for the federal listing. As a result, a multiyear research project on health and diseases of wild desert tortoises was established in California between 1990 and 1995 (Christopher et al., 1999, 2003). Tortoises with clinical signs of possible herpesvirus infection were observed during this study. Several tortoises at two of the three study sites had oral lesions suggestive of the diphtheritic plaques previously described in Mediterranean tortoises (spur-thighed [Greek] tortoise [*Testudo graeca*] and Hermann's tortoise [*Testudo hermanni*]) with herpesvirus infection (Origgi et al., 2004).

Here we summarize previously obtained serologic data, which indicated a widespread distribution of antibodies that bind to TeHV3 isolates in wild populations of desert tortoises in California; report serologic data and DNA results from a repository of necropsied wild tortoises; and provide the first molecular evidence for natural herpesvirus infections in two wild desert tortoises from the Mojave Desert of California and Nevada, USA.

MATERIALS AND METHODS

Animals, studies, and study areas

The animals were from several studies and study areas. We placed our data sets into three groups: 1) serology of captive tortoises from clinics and wild tortoises collected between 1998 and 2002 from sites in the Mojave and Colorado deserts of California; 2) serology and DNA evidence from a repository of 50 salvaged and necropsied tortoises, primarily from California; and 3) molecular evidence from two wild tortoises that died from trauma in 2009 and 2010.

Serology of captive and wild tortoises (Group 1): As part of research projects on health and diseases of tortoises in California, blood samples were obtained from captive and wild

tortoises at multiple sites in the Mojave Desert over several years (e.g., Berry and Christopher, 2001; Berry et al., 2006; Table 1 and Fig. 1). Aliquots of plasma collected from 55 captive tortoises and 256 wild desert tortoises between 1998 and 2002 (Table 1 and Fig. 1) were tested with an indirect enzyme-linked immunoassay (ELISA). The ELISA was previously developed to detect TeHV3 exposure in Mediterranean tortoises (Origgi et al., 2001) and modified for desert tortoise testing. The sources of the 55 captive tortoises were health clinics held in desert towns, one in the western Mojave (Ridgecrest, Kern County, California) and a second in the southern Mojave Desert (Joshua Tree, San Bernardino County, California). The 20 field sites where the 256 samples were collected between 1998 and 2002 from wild tortoises represented five regions of the Mojave and Colorado (western Sonoran) deserts and three of the five recovery units described for the desert tortoise (US Fish and Wildlife Service, 2011).

We estimated prevalences by modeling the proportion of antibody-positive individuals among tortoises within each region, using generalized linear models with a binomial distribution and logit link function (McCullagh and Nelder, 1989) using PROC GENMOD (SAS Institute, 2007). We analyzed data at the site level rather than aggregating across sites, to account for overdispersion due to potential spatial heterogeneity among sites. We used deviance to estimate the multiplier c by which the variance was inflated (i.e., $c=1$ would indicate no overdispersion). We aggregated data across years because initial analyses at the year level did not reveal additional overdispersion resulting from temporal heterogeneity. We tested differences in prevalence among regions (five for wild tortoises and a sixth for all captives) while also testing for effects due to age and sex (adult males, adult females, and juveniles). If age and sex were not significant at the 0.05 significance level, we excluded them from the model for purposes of model parsimony when calculating standard errors and 95% confidence intervals for prevalences in the six regions.

Repository of necropsied tortoises (Group 2): Between 1992 and 2000, 50 salvaged wild desert tortoises were sent to the University of Florida for pathologic evaluation. Following the development of the ELISA for determining TeHV3 exposure, all the tortoises in this group for which ultrafreezer-maintained (−70 C) serum samples were available ($n=27$) were tested for anti-TeHV3 antibodies. If formalin-fixed paraffin-embedded (FFPE) brain tissue was available for the antibody-positive necropsied tortoises, sections were obtained. The PCR tests that were directed to the amplification of the partial sequence of UL39 gene of TeHV3 were performed according to an established protocol (Origgi et al., 2004).

Two recently necropsied tortoises (Group 3): Two adult males were tested for herpesvirus using PCR amplification and nucleic acid sequencing of products that were of appropriate size for targeted genes of herpesvirus. Tortoise 1 (DT 2009 Nec1) was collected 25 September 2009 on the Fort Irwin Road in the central Mojave Desert, 14 km NNE of Barstow, San Bernardino County, California (34°59′56.4″N, 116°55′41.257″W). The tortoise, with a carapace length at the midline (MCL) of 273 mm and weight of 3,759 g, died within minutes of salvage of a major crush injury to its caudal carapace consistent with vehicular trauma. It was a newly observed tortoise with no prior field history. It was submitted frozen to the University of Florida for pathologic evaluation. Tortoise 2 (DT103), with an MCL of 249 mm and weight of 2,120 g, had been part of a research program since 11 September 2007 in the River Mountains of the northeastern Mojave Desert, Clark County, Nevada (36°0′35.64″N, 114°53′2.0394″W). The tortoise had been attacked by a coyote 2 wk prior to collection on 2 November and sustained puncture wounds to limbs and damage to the posterior carapace. At the time of salvage, it was found lying on its carapace, with a coyote nearby, was righted by the field observer but was found dead 1.5 hr later. It was immediately submitted to the Desert Tortoise Conservation Center in Las Vegas, Nevada, for necropsy. Necropsies were performed on both tortoises, and tissues from all major organs were examined by light microscopy according to previously described protocols (Homer et al., 1998).

Indirect ELISA

An indirect ELISA previously developed to detect TeHV3 in Mediterranean tortoises (Origgi et al., 2001) was used to detect serologic reactors in desert tortoises. The TeHV2 antigen could not be used at that time since it was yet to be discovered and the diversity of tortoise herpesviruses was not known. Briefly, two distinct TeHV3 isolates (HV1976 and HV4295/7R/95) were used as antigens, and a tortoise was considered positive when its serum or plasma reacted

TABLE 1. Results for captive and wild desert tortoises (*Gopherus agassizii*) from the Mojave and Colorado deserts, California, USA, tested by enzyme-linked immunosorbent assay (ELISA) for Testudinid herpesvirus 3 (TeHV3) exposure. The tortoises were tested both against HV1976 and HV4295/7R/95 strains of TeHV3 and were considered positive only if the serum of the tested individual reacted against both antigens (see Methods).

General location and status of tortoises	Geographic coordinates	Year	*n*	No. of ELISA-positive tests		
				4295	1976	4295 and 1976
Captive health clinics						
Ridgecrest	35°37′21.0″N, −117°40′14.987″W	1998	27	5	3	3
Joshua Tree/29 Palms	34°7′36.983″N, −116°19′5.016″W	2000	28	9	13	9
Wild Populations						
Western Mojave Desert						
Fremont Valley	35°22′33.924″N, −117°43′41.736″W	2001	1	0	0	0
Desert Tortoise Natural Area	35°11′34.332″N, −117°52′13.548″W	2002	18	5	4	4
Fremont-Kramer	34°47′6.9714″N, −117°20′8.988″W	2002	9	6	6	6
Central Mojave Desert						
Superior-Cronese	35°7′12.9714″N, −116°52′23.9874″W	2001	6	0	0	0
Superior-Cronese	35°7′12.9714″N, −116°52′23.9874″W	2002	6	5	5	5
Ft. Irwin Study Site	35°7′42.1674″N, −116°29′22.632″W	2002	3	2	2	2
Soda Mtns, Ft. Irwin	35°19′51.996″N, −116°17′51.9714″W	2001	10	0	0	0
Tiefort, Ft. Irwin	35°18′58.968″N, −116°28′29.9994″W	1998	7	0	0	0
Tiefort, Ft. Irwin	35°18′58.968″N, −116°28′29.9994″W	2000	6	1	1	1
Tiefort, Ft. Irwin	35°18′58.968″N, −116°28′29.9994″W	2001	14	2	1	1
Eastgate, Ft. Irwin	35°21′59.976″N, −116°22′0.9834″W	1998	9	0	1	0
Southern Mojave Desert						
Lucerne Valley	34°34′12″N, −116°50′44.988″W	2002	6	1	1	1
Ord-Rodman	34°40′29.9994″N, −116°33′15.9834″W	2001	11	2	3	2
Ord-Rodman	34°40′29.9994″N, −116°33′15.9834″W	2002	14	8	8	8
Sand Hill	34°16′21″N, −116°14′10.9674″W	2001	19	4	5	3
Sand Hill	34°16′21″N, −116°14′10.9674″W	2002	21	11	7	7
Bullion	34°19′39″N, −115°49′55.992″W	2002	6	3	3	3
Lavic	34°26′6.972″N, −116°1′35.976″W	2002	8	2	1	1
East Mojave						
Shadow Valley	35°31′13.908″N, −115°44′15.4674″W	2002	2	2	2	2

TABLE 1. Continued.

General location and status of tortoises	Geographic coordinates	Year	*n*	No. of ELISA-positive tests		
				4295	1976	4295 and 1976
Ivanpah Valley 1	35°21′19.98″N, −115°22′4.3674″W	2001	14	1	1	1
Ivanpah Valley 2	35°18′16.992″N, −115°25′13.98″W	2002	25	21	21	21
Fenner	34°55′3.9714″N, −115°19′26.976″W	2002	2	1	0	0
Colorado Desert						
Upper Ward Valley	34°40′12.8634″N, −114°53′36.312″W	2001	8	0	0	0
Upper Ward Valley	34°40′12.8634″N, −114°53′36.312″W	2002	4	0	0	0
Chemehuevi Valley	34°49′45.948″N, −114°57′5.004″W	2002	6	2	2	2
Chocolate Mountains	33°33′6.984″N, −115°34′57.9714″W	2002	21	9	9	9

against both isolates. Cutoff values were set at an optical density (OD) of 0.5 based on the study in Mediterranean tortoises. Final OD values were those obtained following subtraction of the background (mean OD reading of 10 random sera of desert tortoises when tested against uninfected TH-1 cell lysate; TH-1 cells were those used to grow TeHV-3 [ATCC-CCL

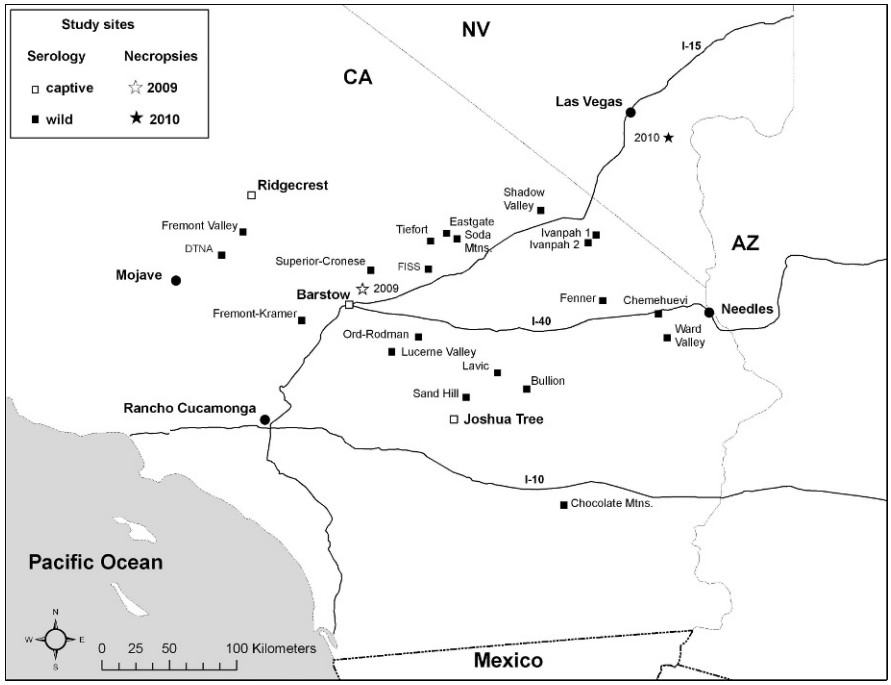

FIGURE 1. Distribution of two necropsied desert tortoises (necropsy 1, 2009; necropsy 2, 2010) and sites in the Mojave and Colorado deserts of California (CA) and Nevada (NV) where 256 wild and 55 captive tortoises were tested for herpesvirus using an enzyme-linked immunosorbent assay for Testudinid herpesvirus 3.

50 Sub-line B1, Rockville, Maryland, USA]). Monoclonal antibody HL 1546 (Origgi et al., 2001) directed against Mediterranean tortoise IgY was replaced by HL 673 (Schumacher et al., 1993) directed against desert tortoise IgY.

Polymerase chain reaction and DNA sequencing

For Group 2 tortoises that were antibody positive to the indirect ELISA, DNA was extracted from FFPE brain using the Qiagen DNeasy kit (Qiagen, Valencia, California, USA), and PCR amplification of a partial sequence of the herpesvirus ribonucleotide reductase large subunit (UL39) gene was performed as described (Origgi et al., 2004). The PCR products were resolved in agarose gels, excised, and purified using the QIAquick gel extraction kit (Qiagen). Products were sequenced directly in both directions using the Big-Dye Terminator Kit (Applied Biosystems, Foster City, California, USA) and analyzed on an ABI 377 automated DNA sequencer at the University of Florida Center for Mammalian Genetics Sequencing Center.

The two fatally injured desert tortoises (Group 3: tortoises 1 and 2) were analyzed independently by separate research teams. For tortoise 1, DNA was extracted from frozen tongue using the Qiagen DNeasy kit (Qiagen). Nested PCR amplification of a partial sequence of the herpesvirus DNA-dependent DNA polymerase gene was performed using methods previously described (VanDevanter et al., 1996). The PCR products were sequenced as above. Primer sequences were edited out prior to further analysis.

For tortoise 2, DNA was extracted from a portion of the tongue and nasal mucosa using Qiagen DNeasy kit (Qiagen). A PCR targeting a 334 base pair (bp) segment of the UL39 gene (GenBank accession DQ027825) with forward primer (5′-GATGCGTGGGATAATGTCGG-3′) and reverse primer (5′-TCGGAGGGAA TGTCTGGAAAC-3′) was performed using an initial denaturation step of 95 C for 5 min followed by 40 cycles of 95 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec, and a 5-min 72 C extension. Next, 100–300 ng of DNA were added to a 25-µl final volume mix containing 25 pmol of each primer, 10 mM Tris (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, and 200 µM each dNTP, and AmpliTaq Gold DNA polymerase (Applied Biosystems) at a final concentration of 0.05 U/µl. The PCR products were resolved in 1% agarose gels, excised, purified using a gel extraction column (Millipore, Billerica, Massachusetts, USA), and directly sequenced in both directions on an ABI 3730 analyzer.

RESULTS

Enzyme-linked immunosorbent assay

For Group 1 tortoises, 12 of 55 (22%) captive tortoises were ELISA positive against both TeHV3 isolates (Table 2 and Fig. 1). Overall prevalence of wild tortoises with positive serology was 31% and varied by region: 36% (10 of 28 tortoises) for the Western Mojave Desert, 15% (9 of 61) for the Central Mojave Desert, and 29% (25 of 85) for the Southern Mojave Desert. Further to the east and south, prevalence was 56% (24 of 43 tortoises) in the Eastern Mojave Desert and 28% (11 of 39) in the Colorado Desert. Within regions, antibody prevalences (where sample sizes were ≥20) ranged from 33% for Sand Hill in 2002 to 84% for Ivanpah Valley in 2002. Despite these variations, no statistical differences existed among regions ($F_{5,38}=1.98$; $P=0.10$) or among age and sex classes ($F_{2,38}=1.36$; $P=0.27$). The lack of significant difference is partly attributed to overdispersed variation ($c=2.3$). For example, the site-specific prevalence among tortoises from the East Mojave region ranged from 7% (1/14 at Ivanpah Valley 1; Table 1) to 84% (21/25 at Ivanpah Valley 2). The standard errors and confidence intervals reflect relatively low precision in regional estimates, although an emerging pattern suggests prevalence rates are high in the East Mojave and low in the Central Mojave Desert (Table 2).

Of the 50 Group 2 tortoises necropsied between 1992 and 2000, sera were available for 27 tortoises for testing with the ELISA for antibody against TeHV3. Six of the 27 tortoises were positive for both TeHV3 isolates.

Polymerase chain reaction and DNA sequencing

Of the six Group 2 tortoises that were positive by TeHV3 ELISA, FFPE brain was available for PCR testing from two tortoises (DT50 from Chemehuevi Valley and DT55 from Goffs; both in San Bernardino County, California). They were necropsied in 1999 and 2000, respectively. For both tortoises, the PCR

TABLE 2. Estimated prevalence, standard errors (SE), and 95% confidence intervals for captive and wild desert tortoises (*Gopherus agassizii*) from the Mojave and Colorado deserts, California, USA, positive for antibody to Testudinid herpesvirus 3 by enzyme-linked immunosorbent assay.

Region	Estimated prevalence (%)	SE (%)	Confidence interval (lower, upper)
Clinics (captive)	22	9	9, 43
Western Mojave	36	14	14, 65
Central Mojave	15	7	5, 34
Southern Mojave	29	8	17, 46
Eastern Mojave	56	12	33, 76
Colorado Desert	28	11	12, 54

yielded a product of the expected size (386 bp; Origgi et al., 2004). However, the results from sequencing of the amplicons were inconclusive.

For tortoise 1 in Group 3, PCR of the tongue resulted in a product that consisted of 181 bp. When compared to known sequences in GenBank (National Center for Biotechnology Information, Bethesda, Maryland), EMBL (Cambridge, United Kingdom), and Data Bank of Japan (Mishima, Shizuoka, Japan) using BLASTN (Altschul et al., 1997), there was 100% identity of the sequences with the DNA-dependent DNA polymerase gene of TeHV2 (GenBank accession AY916792). The tongue and the nasal mucosa of tortoise 2 were positive for the UL39 gene of TeHV2 with PCR. Amplicons (334 bp) from PCR had 100% nucleotide identity with the UL39 gene of TeHV2 (GenBank accession DQ027825).

Pathologic findings

In the two Group 2 necropsied desert tortoises (DT50, DT55) that were antibody positive by the TeHV3 ELISA (DT50 and DT55), no macroscopic or microscopic lesions similar to those normally observed in tortoises infected with TeHVs (Origgi et al., 2004) were seen. Tortoise 1 (Group 3) had moderate atrophy of skeletal musculature with no fat associated with pectoral and pelvic girdles. There was atrophy of the right and left thymus and atrophy of the liver, which was dark in color and weighed 83.7 g (2.2% of body weight). Although freezing resulted in substantial artifactual change to all tissues, most tissues were of sufficient quality to note moderate to severe histologic change. Light microscopic examination of all major tissues identified no abnormalities. Compared to livers of most previously necropsied tortoises, there was atrophy of hepatocytes; no vacuolization was seen. There was a concomitant moderate increase in the number of melanomacrophages per low-power field in both lobes of the liver.

Necropsy of tortoise 2 (Group 3) revealed that both hind legs had proximal, focally extensive nonhealed lacerations of the skin and corresponding skeletal muscle. There was mild atrophy of skeletal musculature with small fat deposits at the pectoral and pelvic girdles. The liver had an absolute weight of 66.4 g (3.1% of the total body weight). It had moderate, diffuse vacuolization of hepatocytes and moderate numbers of melanomacrophages. Other incidental findings included occasional protozoal cysts (*Sarcocystis* sp.) in the skeletal musculature and focal, small foreign body granuloma in the tongue and stomach.

DISCUSSION

We present the first conclusive evidence of infection of two wild desert tortoises with TeHV2. This complements previous serologic surveys undertaken on captive (Johnson et al., 2006) and wild tortoises (Origgi, unpubl.) in California between 1998 and 2002 using an indirect ELISA developed for Mediterranean tortoises (Origgi et al., 2001) and modified for use in desert tortoises. During 2000–01, Johnson et al.

(2006) tested a large sample of captive desert tortoises from the same general area using this indirect TeHV3 ELISA and reported that 26.6% were antibody positive. The serologic data from an additional 55 captive and 256 wild desert tortoises from 20 sites (Group 1, Table 1) and two (Group 2) necropsied wild tortoises were strongly suggestive that wild desert tortoises throughout the deserts of California had antibodies reactive against a tortoise herpesvirus. Nevertheless, no other supportive evidence was available. The positive PCR results obtained from two Group 2 antibody-positive tortoises could not be confirmed by sequencing, and no classic TeHV-associated lesions were seen in the tissues of those tortoises. At the time the serologic work was undertaken, TeHV2 was yet to be discovered and its relationship with TeHV3 was unknown. Further, the ELISA had been validated for TeHV3 (not known to be distinct at that time) in Mediterranean tortoises but not in desert tortoises. Some of these concerns were answered when Johnson et al. (2005) reported the identification of TeHV2 as a novel herpesvirus infecting a captive desert tortoise. Using the TeHV3 ELISA described above, seroconversion was detected in the infected tortoise. The most probable explanation for this is cross-reactivity between TeHV2 and TeHV3. Because of this, and until TeHV2 is isolated or recombinant antigen is expressed, we believe that TeHV3 may be used as a surrogate antigen for antibody-prevalence studies of nonspecific antiherpesvirus antibodies in desert tortoises.

The failure to sequence the TeHV2 UL39 gene in the two Group 2 desert tortoises that were ELISA positive for antiherpesvirus antibodies may be partially explained by nucleotide differences between the UL39 gene of TeHV3 and the homologous gene of TeHV2, resulting in weaker primer binding and loss of assay sensitivity. However, this protocol has been previously used successfully to amplify the UL39 gene of TeHV2 from frozen tissues (Johnson et al., 2005). The use of FFPE tissues may have also played a significant role in lack of sensitivity; FFPE tissues are inferior to frozen tissues for PCR amplification of other DNA viruses of reptiles (Garner et al., 2008).

Our serologic findings indicate that TeHV2 occurs in wild desert tortoises in different areas of the Mojave and Colorado deserts and may be sufficiently related to cross-react with TeHV3 in an indirect ELISA. Given the apparent high antibody prevalence, the ability of herpesviruses to establish latent infections in endemic hosts, and the comparatively low disease rate seen with herpesviruses in desert tortoises, it is probable that the infection rate is significantly higher than the disease rate. Herpesvirus infections often cause subclinical or mild disease in endemic host species, in which they establish latency, and fatal disease in aberrant species. There are numerous examples of herpesviruses causing more severe disease in nonnative hosts (Ostrowski et al., 1998; Dunowska et al., 2001; Landolfi et al., 2005; Pinkerton et al., 2008). Relatively small phylogenetic differences may be highly clinically significant. Human herpesvirus 1 and Macacine herpesvirus 1 (McHV1) are more closely related than TeHV2 and TeHV3 (Bicknese et al., 2010). Human herpesvirus 1 primarily causes asymptomatic infections or occasional mild cold sores in humans, whereas McHV1 is rapidly fatal in humans. It is reasonable to hypothesize that similar clinical differences may exist for different tortoise herpesvirus species in different tortoise host species.

The apparent cross-reactivity of TeHV2 and TeHV3 antibodies illustrates an important confounding factor in infectious disease diagnostics in species for which potential pathogens are not well characterized. Specific pathogen identification is crucial in any diagnostic assay, and the potential for yet unknown cross-reactive agents is significant in these species. In a recent study using a consensus PCR

(Martel et al., 2009), 16% of clinically healthy tortoises of diverse species that were randomly sampled for tortoise herpesvirus had bands of the appropriate size. However, no sequence identification of any products was done, and the protocol used in that study amplified all known viruses in the proposed genus *Chelonivirus*. Treating diverse herpesviruses as a single entity may lead to inappropriate management decisions. From a population management perspective, it is crucial to know which viruses are endemic in a host species and the pathogenic implications of these viruses in other potential contact species. Understanding of the host ranges of different tortoise herpesvirus species is still preliminary. Testudinid herpesvirus 1, apparently endemic in Russian tortoises (*Agrionemys horsfieldii*), has been identified in separate incidents in diseased pancake tortoises (*Malacochersus tornieri*), Hermann's tortoises, and Agassiz's desert tortoises, all with a history of exposure to Russian tortoises (Une et al., 2000; Stöhr and Marschang, 2010; Wellehan et al., unpubl.).

Our preliminary findings need to be further investigated to understand their biologic and epidemiologic meaning. New field surveys are needed to determine whether there is further diversity of endemic herpesvirus species in the desert tortoise species complex (Murphy et al., 2011), the prevalence of virus and viral antibodies to TeHV2, frequency of TeHV2 shedding, and distribution of the TeHV2. Transmission and pathogenesis studies also are needed. Use of TeHV3 as a surrogate antigen for TeHV2 in the ELISA needs further validation, and development of more specific assays would be preferable. Even more urgent is the need to develop protocols for testing and monitoring desert tortoises expected to be translocated as a mitigation measure to reduce effects on the tortoise from development. Thousands of desert tortoises are being displaced because of the expansion of military bases and development for renewable energy (Esque et al., 2005; Independent Science Advisors, Desert Renewable Energy Conservation Plan, 2010). Translocated tortoises need to be closely monitored, because this is a time when they are often stressed and consequently are likely to exhibit active infection or shed virus.

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